

PATENT COOPERATION TREATY

From the

INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

ON XL
ON DB

To:

ROLLINS, Anthony J.
NYCOMED AMERSHAM PLC
Amersham Laboratories
White Lion Road
Amersham
Buckinghamshire HP7 9LL
GRANDE BRETAGNE

DATE	2/11/01
FORMAL REPLY	SH ✓
INT. OFF.	PR
ON DB	6/11/01
CASE NO.	PB9944 WO

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing
(day/month/year)

31.10.2001

Applicant's or agent's file reference
PB-9944-PCT

IMPORTANT NOTIFICATION

International application No.
PCT/US00/22150

International filing date (day/month/year)
10/08/2000

Priority date (day/month/year)
21/08/1999

Applicant

AMERSHAM PHARMACIA BIOTECH INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

 European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Cleere, C

Tel. +49 89 2399-7713



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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PB-9944-PCT	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/22150	International filing date (<i>day/month/year</i>) 10/08/2000	Priority date (<i>day/month/year</i>) 21/08/1999
International Patent Classification (IPC) or national classification and IPC C12N15/54		
Applicant AMERSHAM PHARMACIA BIOTECH INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 4 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 07/03/2001	Date of completion of this report 31.10.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Meyer, W Telephone No. +49 89 2399 8157 

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**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US00/22150

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-22 as originally filed

Claims, No.:

1-28 as originally filed

Drawings, sheets:

1-19 as originally filed

Sequence listing part of the description, pages:

1-12, filed with the letter of 31.12.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

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**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US00/22150

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-25
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-25
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-25
	No:	Claims	

- 2. Citations and explanations
see separate sheet**

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

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Re V:

1. The documents mentioned in this written opinion are numbered as in the International Search Report (ISR), i.e. D1 corresponds to the first document of the ISR etc.

2. With respect to the documents cited in the International Search Report, the claimed subject-matter of **claims 1, 2, 15 and 16** fulfill the requirement of Article 33(2) PCT.

The subject-matter of **claims 1, 2, 15 and 16** also fulfill the requirement of Article 33(3) PCT:

Document D3 is considered to represent the closest prior art document, which discloses a DNA polymerase having 540-582 amino acids and a tyrosine at a position equivalent to position 667 of Taq DNA polymerase, lacking 5' to 3' exonuclease. **Claims 1, 2, 15 and 16** are distinguished therefrom by disclosing the E681R DNA polymerase and variants thereof. The technical problem to be solved by the present application was therefore to provide an alternative Taq DNA polymerase. The solution to this problem proposed in **claims 1, 2, 15 and 16** of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons: Although there are several different variations of Taq DNA polymerase known to the skilled person at the relevant date. However, a person skilled in the specific field could not directly deduce in an obvious manner from the available prior art that the claimed specific Taq DNA polymerase has a substantial improvement of signal uniformity compared to Taq Delta 271/F272M/F667Y DNA polymerase.

Consequently, the subject-matter of present **claims 1, 2, 15 and 16** fulfill the requirements of Article 33(3) PCT.

The remaining **claims 3-14 and 17-22** are depended on or referring back to the subject-matter of **claims 1, 2, 15 and 16** and fulfill thus also the requirement of Article 33 (2) and Article 33(3) PCT.

Re VIII:

3. The subject-matter of present **claims 2 and 15 or 3 and 16** are superfluous (Article 6 EPC).

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PATENT COOPERATION TREATY

RECEIVED

FROM THE INTERNATIONAL SEARCHING AUTHORITY

NOV 21 2000
 AMERSHAM PHARMACIA BIOTECH INC.
 Attn: VICTORIA, M.M.
 800 Centennial Avenue
 ROYAL L. BONNING, JR.
 P.O. Box 1327
 Piscataway, NJ 08855-1327
 UNITED STATES OF AMERICA

PCT

NOTIFICATION OF TRANSMITTAL OF
 THE INTERNATIONAL SEARCH REPORT
 OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing (day/month/year) 20/11/2000	
Applicant's or agent's file reference PB-9944	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US 00/ 22150	International filing date (day/month/year) 10/08/2000
Applicant AMERSHAM PHARMACIA BIOTECH INC. et al.	

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
 34, chemin des Colombettes
 1211 Geneva 20, Switzerland
 Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentlaan 2
 NL-2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Mireille Claudepierre

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NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of the filing of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

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The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference PB-9944	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 00/ 22150	International filing date (day/month/year) 10/08/2000	(Earliest) Priority Date (day/month/year) 21/08/1999
Applicant AMERSHAM PHARMACIA BIOTECH INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☒ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☒ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

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☐ None of the figures.

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 00/22150

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 7	C12N15/54 C12P19/34	C12N15/70 //(C12N1/21, C12R1:19)
C12N9/12	C12N1/21	C12Q1/68
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 7 C12N C12Q C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
WPI Data, PAJ, CAB Data, STRAND, EPO-Internal		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 902 035 A (HOFFMANN LA ROCHE) 17 March 1999 (1999-03-17) page 6, line 21 - line 28; tables I, II	
A	WO 98 40496 A (PERKIN ELMER CORP) 17 September 1998 (1998-09-17) claims 1-15	
A	EP 0 745 676 A (AMERSHAM LIFE SCIENCE INC) 4 December 1996 (1996-12-04) the whole document	
A	US 5 885 813 A (DAVIS MARIA ET AL) 23 March 1999 (1999-03-23) the whole document	
	-/--	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
13 November 2000		20/11/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Hornig, H

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INTERNATIONAL SEARCH REPORT

national Application No

T/US 00/22150

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EP 0 655 506 A (HARVARD COLLEGE) 31 May 1995 (1995-05-31) the whole document</p> <p>-----</p>	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/22150

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0902035	A	17-03-1999	AU 8416198 A	25-03-1999
			BR 9803419 A	08-02-2000
			CA 2243985 A	11-03-1999
			CN 1218832 A	09-06-1999
			CZ 9802874 A	17-03-1999
			HU 9802024 A	28-05-1999
			JP 11137284 A	25-05-1999
			NO 984157 A	12-03-1999
			PL 328525 A	15-03-1999
WO 9840496	A	17-09-1998	AU 6466998 A	29-09-1998
			EP 0983364 A	08-03-2000
EP 0745676	A	04-12-1996	CA 2222744 A	05-12-1996
			GB 2301590 A,B	11-12-1996
			JP 11507211 T	29-06-1999
			WO 9638568 A	05-12-1996
			US 5885813 A	23-03-1999
US 5885813	A	23-03-1999	CA 2222744 A	05-12-1996
			EP 0745676 A	04-12-1996
			GB 2301590 A,B	11-12-1996
			JP 11507211 T	29-06-1999
			WO 9638568 A	05-12-1996
EP 0655506	A	31-05-1995	US 5614365 A	25-03-1997
			AT 143057 T	15-10-1996
			AU 4193396 A	06-05-1996
			CA 2201885 A	25-04-1996
			CN 1170439 A	14-01-1998
			DE 29513622 U	19-10-1995
			DE 29513639 U	19-10-1995
			DE 69400567 D	24-10-1996
			DE 69400567 T	06-02-1997
			DE 655506 T	28-09-1995
			DK 655506 T	10-03-1997
			EP 0727496 A	21-08-1996
			ES 2072238 T	16-07-1995
			FI 971611 A	13-06-1997
			GR 95300040 T	31-07-1995
			HU 77037 A	02-03-1998
			JP 2691149 B	17-12-1997
			JP 8205874 A	13-08-1996
			WO 9612042 A	25-04-1996
			ZA 9508761 A	13-05-1996

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PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE
in its capacity as elected Office

Date of mailing (day/month/year)
25 April 2001 (25.04.01)

International application No.
PCT/US00/22150

Applicant's or agent's file reference
PB-9944

International filing date (day/month/year)
10 August 2000 (10.08.00)

Priority date (day/month/year)
21 August 1999 (21.08.99)

Applicant

DAVIS, Maria et al

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
07 March 2001 (07.03.01)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Mougamadou ABIDINE

Telephone No.: (41-22) 338.83.38

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(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
1 March 2001 (01.03.2001)

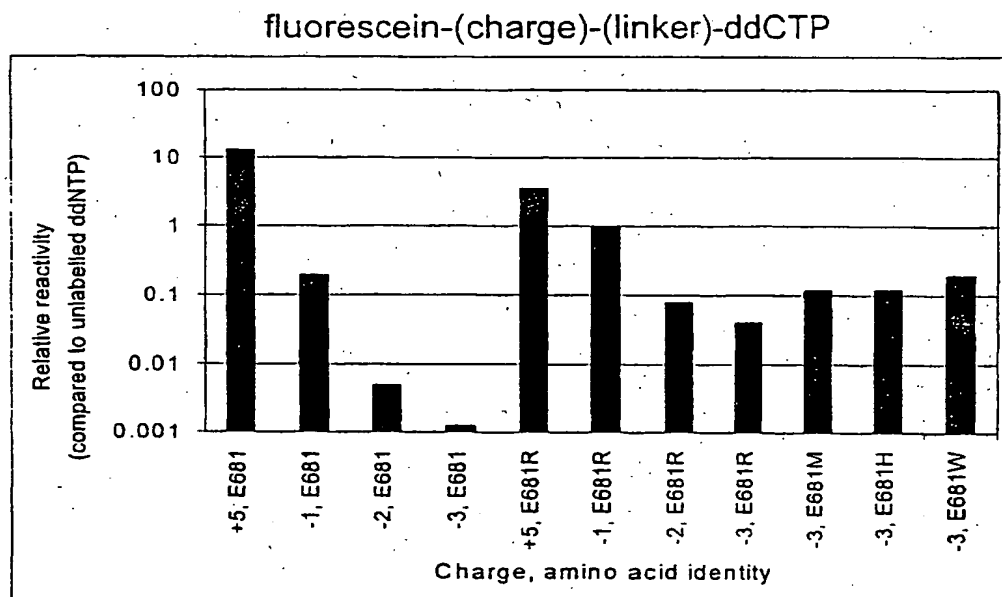
PCT

(10) International Publication Number
WO 01/14568 A1

- (51) International Patent Classification⁷: C12N 15/54. 15/70, 9/12, 1/21, C12Q 1/68, C12P 19/34 // (C12N 1/21, C12R 1:19)
- (21) International Application Number: PCT/US00/22150
- (22) International Filing Date: 10 August 2000 (10.08.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/150,167 21 August 1999 (21.08.1999) US
60/154,739 17 September 1999 (17.09.1999) US
- (71) Applicant (for all designated States except US): AMER-
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(54) Title: Taq DNA POLYMERASES HAVING AN AMINO ACID SUBSTITUTION AT E681 AND HOMOLOGS THEREOF
EXHIBITING IMPROVED SALT TOLERANCE



(57) Abstract: Thermostable DNA polymerases having an E410R substitution which result in a substantial improvement of signal uniformity compared to Taq $\Delta 271/F272M/F667Y$ DNA polymerase. The instant DNA polymerases possess improved salt tolerance and have been shown to modulate the incorporation of terminators having a net positive or a net negative charge during the sequencing reaction.



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TAQ DNA POLYMERASES HAVING AN AMINO ACID SUBSTITUTION AT E681
AND HOMOLOGS THEREOF EXHIBITING IMPROVED SALT TOLERANCE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 USC § 119(e) of US provisional application serial number 60/150,167, filed on August 21, 1999, and US provisional application serial number 60/154,739, filed on September 17, 1999, the entire disclosures of each of which are incorporated in their entirety herein.

BACKGROUND OF THE INVENTION

Field of the Invention

The instant disclosure pertains to thermostable DNA polymerases which exhibit improved robustness and efficiency. In particular, the instant DNA polymerase has been shown to result in a substantial improvement of signal uniformity compared to Taq Δ 271/F272M/F667Y DNA polymerase when used in DNA sequencing reactions.

Background

DNA polymerases are enzymes which are useful in many recombinant DNA techniques such as nucleic acid amplification by the polymerase chain reaction ("PCR"), self-sustained sequence replication ("3SR"), and high temperature DNA sequencing. Thermostable polymerases are particularly useful. Because heat does not destroy the polymerase activity, there is no need to add additional polymerase after every denaturation step.

Naturally occurring DNA polymerases preferentially incorporate unlabeled nucleotides over corresponding labeled nucleotides into polynucleotides. This ability of DNA polymerases to discriminate against fluorescently labeled nucleotides had an undesirable effect on many molecular biology procedures that require the enzymatic addition of labeled nucleotides, e.g., labeled dideoxy terminator sequencing. Ambiguous sequencing determinations often result from the disproportionate number of labeled and unlabeled dideoxy terminators and nucleotides. On an electropherogram obtained from a capillary

electrophoresis sequencing unit, this phenomena shows up as uneven peaks. Large signals due to a larger amount of incorporated labeled ddNTP (shown as wide peaks) can obscure smaller signals and lead to ambiguous sequence determinations. Additionally, many of the enzymes presently available are sensitive to high salt environments.

Thus, a need continues to exist for an improved DNA polymerase having improved discrimination properties (and thus resulting in improved signal uniformity) and increased tolerance to high salt conditions. These and other concerns are addressed in greater detail below.

BRIEF SUMMARY OF THE INVENTION

The instant disclosure teaches a purified recombinant thermostable DNA polymerase comprising the amino acid sequence set forth in Figure 2 or 3. The instant disclosure also teaches an isolated nucleic acid that encodes a thermostable DNA polymerase, wherein said nucleic acid consists of the nucleotide sequence set forth in Figure 2 or 3, as well as a recombinant DNA vector that comprises the nucleic acid, and a recombinant host cell transformed with the vector. The instant disclosure also teaches a method of sequencing DNA comprising the step of generating chain terminated fragments from the DNA template to be sequenced with the DNA polymerase in the presence of at least one chain terminating agent having a net negative or a net positive charge and one or more nucleotide triphosphates, and determining the sequence of said DNA from the sizes of said fragments. The instant disclosure also teaches a kit for sequencing DNA comprising the DNA polymerase and nucleic acid terminator having a net negative or a net positive charge.

DETAILED DESCRIPTION

One objective of the instant disclosure is to increase the uniformity of dye-terminator incorporation in fluorescent dye DNA sequencing. One important DNA polymerase is Taq DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*, the amino acid sequence for which is shown at Figure 1. The full length enzyme was truncated to eliminate 5' to 3' exonuclease activity and to provide a polypeptide more stable to proteolysis and heat treatment. The truncated enzyme is known as Taq $\Delta 271$ /F272M/F667Y DNA polymerase, which is commercially available from Amersham Pharmacia Biotech as Thermo Sequenase[®] DNA polymerase. Position 1 (amino acid Met) in Taq $\Delta 271$ /F272M/F667Y

DNA polymerase corresponds to position 272 in full length Taq polymerase. It should be noted that the numbering used in the instant disclosure is that for Taq $\Delta 271$ / F272M/F667Y polymerase, not for Taq polymerase.

Single amino acid substitutions were introduced into Taq $\Delta 271$ / F272M/F667Y polymerase. These substitutions are designated as E344Q, I367V, F367Y, E416K and E410R. Each of the substituted polymerases was expressed, purified, and analyzed for uniformity of dye-terminator incorporation in fluorescent sequencing studies, as assayed by signal uniformity. The E410R substitution was found to result in a substantial improvement of signal uniformity compared to Taq $\Delta 271$ / F272M/F667Y DNA polymerase.

The DNA polymerases disclosed herein are especially suitable for use in sequencing reactions which employ terminators having a net positive or a net negative charge. Surprisingly, the instant DNA polymerases have been shown to modulate the incorporation of such terminators during the sequencing reaction. See for example Figure 14. Furthermore, such nucleic acid terminators, which along with the corresponding nucleic acid terminator decomposition products, migrate on separation media at different rates than the sequencing reaction products and which result in improved sequence data. These nucleic acid terminators also allow for the direct loading of nucleic acid sequencing reactions onto separating media. To achieve this goal, negatively or positively charged moieties are attached to the terminator molecule. The unreacted or degraded terminators containing such charged moieties move faster (negatively charged) or in the reverse direction (positively charged) than the DNA sequencing products.

For example, the structures depicted in Figure 15 illustrate potential sites at which a charged moiety may be attached to a terminator. Referring to Figure 15, the Base may comprise A, T, G, C or analogs such as 7-deazapurine, inosine, universal bases. The Sugar may comprise furanose, hexose, mono-di-triphosphates, morpholine, didehydro, dideoxyribose, deoxyribose. The Linker may comprise 1-100 atoms, preferably 2-50 atoms consisting of C, H, N, O, S and halogens. The Mobility modifier may comprise any charged species which alters electrophoretic mobility of structure and degradation products, e.g., α -sulfo- β -alanine, cysteic acid, sulfonic acids, carboxylates, phosphates, phosphodiesteres, phosphonates, amines, quaternised amines, and phosphonium moieties. The Mobility modifier may comprise a number of these units covalently linked together. The Label may comprise any signal moiety such as radioisotope, electrochemical tag, fluorescent tags,

energy transfer (ET) labels, mass spectrometry tags, Raman tags, hapten, chemiluminescent group, enzyme, chromophore, and two or more labels. The label may also be charged, e.g. Cy5.5, bis-sulfonated carboxyfluorescein, or a dye attached to a charged moiety, e.g., carboxyfluorescein attached to cysteic acid or similar charged species. Methods for making these and other compounds are disclosed in U.S. Provisional Application No. 60/098,469 filed on August 31, 1998, and U.S. Application No. 90/018,695 filed on February 4, 1998, and PCT/GB98/00978 filed on April 2, 1998 and published on October 8, 1998, the disclosures of each application are incorporated in their entirety by reference herein.

The following examples are for illustration purposes only and should not be used in any way to limit the appended claims.

EXAMPLES

EXAMPLE 1

The construction, expression and purification of Taq $\Delta 271/F272M/F667Y/E410R$ polymerase is described below. The other substitutions named above were constructed, expressed and purified in a similar manner.

Construction

Primers BamHIFOR (5' ccg ctt ggg cag agg atc cgc cgg gcc ttc atc gcc gag ga) and NheIREV (5' tcg taa ggg atg gct agc cgc tgg gag agg cgg tgg gcc gac) were used in a standard PCR reaction to amplify the region between the BamHI and NheI restriction sites in pREFY2pref (cloned Taq $\Delta 271/F272M/F667Y$ DNA polymerase). Primer BamHIFOR contains a BamHI restriction site which corresponds to the same unique site in pREFY2pref, and primer NheIREV contains a NheI restriction site which corresponds to the same unique site in pREFY2pref. In addition, primer NheIREV was designed to change the codon at position 410 from gag (encoding amino acid E, glutamic acid) to cgg (amino acid R, arginine). The PCR product was digested with the appropriate enzymes, and isolated by agarose gel electrophoresis. The large fragment resulting from the BamHI/NheI digestion of pREFY2pref was also gel purified, and ligated to the PCR fragment above. Following transformation into *E. coli*, plasmid DNA was isolated and subsequently sequenced to confirm the presence of the E410R substitution. The amino acid sequence for Taq $\Delta 271/F272M/F667Y/E410R$ DNA polymerase is shown at Figure 2.

Expression & Purification of the Taq $\Delta 271/F272M/F667Y/E410R$ Polymerase

Vector pRE2 which carries the lambda p_L promoter was used with an *E. coli* strain which has the heat labile repressor protein $cl857$ to express the Taq $\Delta 271/F272M/F667Y/E410R$ polymerase. This combination permits cultivation at 30°C followed by expression of a plasmid-borne protein at elevated temperatures such as 42°C. Liquid cultures were typically grown at 30°C to an OD_{600} of ~ 1.0 , and then transferred to 42°C for ~ 2.5 hours. Bacterial cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.1% Tween-20, 0.1% Triton X-100, 10 mM $MgCl_2$, and 16 mM $(NH_4)_2SO_4$), and then heated at 80°C for 20 minutes to precipitate *E. coli* proteins. The heat lysate was clarified by centrifugation, and supplemented with 300 mM NaCl, and applied to a DE52 anion exchange column (commercially available from Whatman). The flow-through was diluted in Buffer A (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100 and 0.1% Tween-20) to reduce the NaCl concentration to 100 mM, and applied to a Heparin Sepharose column (commercially available from Pharmacia Inc.). The column was developed by linear gradient from 100 to 700 mM NaCl in Buffer A. The enzyme eluted at ~ 250 mM NaCl. Fractions containing polymerase activity were pooled, concentrated on a Centriprep-50 apparatus (commercially available from Amicon) and dialyzed extensively against a final buffer containing 20 mM Tris-HCl pH 8.5, 50% glycerol, 0.1 mM EDTA, 0.5% Tween-20, 0.5% Triton X-100, 100 mM KCl and 1 mM DTT. The purity of the polymerase preparation was confirmed by SDS-PAGE.

Enzyme Characterization

1) Salt tolerance:

The Taq $\Delta 271/F272M/F667Y/E410R$ DNA polymerase activity has been examined under a KCl titration experiment by using both activated salmon sperm DNA and primed M13 DNA as substrates. In both assays, Taq $\Delta 271/F272M/F667Y/E410R$ showed a decreased polymerase activity while increasing KCl concentration from 0 to 200 mM. However, the enzyme displays a much slower activity decrease compared to TS. Figure 4 plots the data from KCl titration of Taq $\Delta 271/F272M/F667Y$ and Taq $\Delta 271/F272M/F667Y/E410R$ using activated salmon sperm DNA as substrates. The 50% KCl inhibition for Taq $\Delta 271/F272M/F667Y/E410R$ polymerase activity with activated salmon sperm or primed

M13 DNA are 120 mM and 100 mM, respectively compared to TS, which has a 50% KCl inhibition of 35 mM. The polymerase assay buffer contains: 25 mM TAPS (pH 9.3), 2 mM MgCl₂, 1 mM 2-mercaptoethanol and 200 mM each dNTP plus 0.05 Ci/mmol [α -³³P]-dATP. A comparison of salt tolerance data for Taq Δ 271/F272M/F667Y and substitutions thereof is presented below in Table I.

TABLE I

<u>Enzyme, substitution</u>	<u>Salt Tolerance</u>
Taq Δ 271/F272M/F667Y	35 mM
Taq Δ 271/F272M/F667Y/E410R	135 mM
Taq Δ 271/F272M/F667Y/E410M	125 mM
Taq Δ 271/F272M/F667Y/E410W	125 mM
Taq Δ 271/F272M/F667Y/E410H	110 mM

2) Thermostability at 95°C:

The thermostability of Taq Δ 271/F272M/F667Y/E410R has been assayed as follows. First, the 95°C heating step was performed in a buffer containing 50 mM Tris-HCl pH 9.5, 5mM MgCl₂, 50 μ M each dNTP and 100ng M13 single strand DNA. Then 10 units of enzyme were mixed with the above solution and a time course performed by taking aliquots (20 μ l each) and placing on ice. Next, dilutions were made in a buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 0.5% Tween-20, 0.5% Nonidet P-40. In the third step, the heated and diluted samples have been assayed for survivor polymerase activity under a standard polymerase assay condition described in section (1) but including 50 mM KCl. Figure 5 showed the thermostability assay of comparing Taq Δ 271/F272M/F667Y/E410R with Amplitaq. The 50% inhibition time at 95°C for Taq Δ 271/F272M/F667Y/E410R and Amplitaq are 25 min and 8min, respectively.

3) Processivity assay:

The processivity of Taq $\Delta 271/F272M/F667Y/E410R$ has been examined in an enzyme dilution method, which insures that the polymerase activity is assayed for a single enzyme binding event. The assay buffer contains 15 mM Tris-HCl (pH 9.5), 3.5 mM $MgCl_2$, 100 mM each dNTP and $1\mu g$ P^{33} labeled primed M13. The primer extension experiment has been performed at $65^\circ C$ for 90 seconds. The samples were analyzed on a 8% polyacrylamide-7 M urea sequencing gel. Taq $\Delta 271/F272M/F667Y/E410R$ has an increased processivity of about 30 nucleotides per polymerase binding event. This is about a 7 to 8 fold increases compared to Taq $\Delta 271/F272M/F667Y$ (4 nt/binding event).

4) Uniform termination events:

The new E to R amino acid modification discovered also results in increased uniformity in termination events during sequencing reactions containing net positive, negative, or neutrally charged dideoxynucleotide terminators. This results in an increased uniformity in electropherogram band intensity and an increase in the number of bases which can be basedcalled per sequence. For example, as shown in Figure 6, the average deviation of band intensity using Thermosequenase Version II is about a 30% deviation. However, as shown in Figure 7, a typical result using an E to R polymerase is about a 22% deviation. This improvement is significant. Portions of Figures 6 and 7 are magnified in Figures 8 through 10 for comparison purposes.

5) Ability to sequence difficult areas:

The new E to R amino acid modification discovered also results in an improved ability to sequence DNA's which contain "difficult to sequence" areas. Certain specific DNA sequences are extremely likely to cause sequencing DNA polymerases problems, resulting in a reduced quality of the sequence obtained (see Figure 11). Surprisingly, enzymes containing the E to R modification are much more likely to yield higher quality sequence data from DNA containing these difficult to sequence areas (see Figure 12).

EXAMPLE 2: TAQ D18A/E681R/F667Y POLYMERASE

We also constructed using standard techniques described above a full length version of Taq polymerase with the following substitutions : D18A/E681R/F667Y. In this enzyme, the D18A substitution removes the 5' to 3' exonuclease activity, rather than the deletion of

amino acids as in the Taq $\Delta 271/F272M/F667Y$ DNA polymerase polypeptide. The E681R substitution is the position equivalent to E410R in Taq $\Delta 271/F272M/F667Y$ DNA polymerase, and F667Y is the equivalent position to F396Y in Taq $\Delta 271/F272M/F667Y$ DNA polymerase. This enzyme also has properties desirable for sequencing with dye terminators. The amino acid sequence of Taq D18A/E681R/F667Y DNA polymerase is shown at Figure 3.

Uniformity of positive terminator reactions is improved considerably with the substitutions at E681 as shown by the data in Table II below.

TABLE II

<u>Enzyme, substitution</u>	<u>Uniformity (r.m.s.)</u>
TSI, E681	0.52
TSI, E681R	0.39
TSI, E681H	0.37
TSI, E681I	0.4
TSI, E681M	0.31
TSI, E681W	0.34

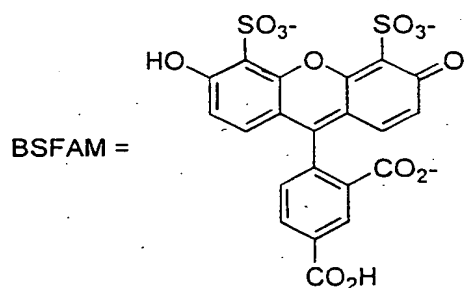
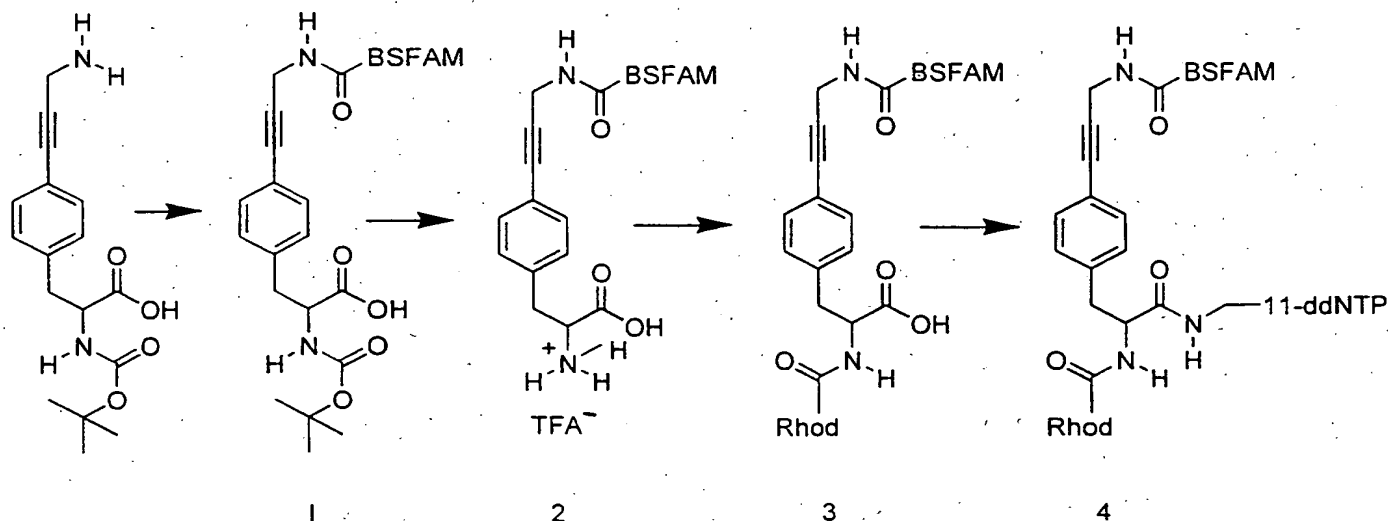
Root mean square ("r.m.s.") is a measure of uniformity of a four color sequence reaction. This experiment used positive terminators (5 lysines in the linker) and standard sequencing reaction conditions. The improvement of 0.52 to below 0.45 shows a significant increase in uniformity for the sequencing reaction.

Figure 13 is a side-by-side comparison of electropherograms obtained from four color sequencing reactions conducted using D18A/F667Y DNA polymerases having various E681 substitutions as described at the left of each electropherogram. As shown in Figure 13, D18A/E681R/F667Y shows the most uniform peak heights and thus the most improvement in uniformity.

Figure 14 shows the relative reactivity compared to unlabelled ddNTPs evidenced in four color sequencing reactions which employed D18A/F667Y and various E681 substitutions thereof with various charged terminators.

Nucleic Acid Terminators1. An example of charge modified reporters as applied to direct load1.1 Chemistry

The following scheme was used to synthesize labeled ddNTPs with a charged reporter moiety. The linker was synthesized according to methods disclosed in U.S. Provisional Application No. 60/098,469 filed on August 31, 1998, the entire disclosure of which is hereby incorporated by reference herein.



Rhod = 5-R110, 5-ROX, 5-TAMRA, 5-REG

1.2 Discussion

4',5' Bis-sulfono-5-carboxyfluorescein (BSFAM) was attached to 4-propargylamino-N- α -*t*-butoxycarbonylphenylalanine by initial formation of the corresponding N-hydroxysuccinimide active ester using TSTU in DMF/diisopropylethylamine. Activation times were typically 15 minutes as observed by tlc before addition of the amino component. The product 1 was isolated by C18 RP-HPLC then treated with neat trifluoroacetic acid to remove the carbamate moiety, with the product 2 isolated by Et₂O precipitation. Attachment of the rhodamine moiety was carried out using 5-rhodamine hydroxysuccinimide active esters in DMSO/diisopropylethylamine. All the double dye cassettes were purified by reverse phase HPLC prior to conjugation to alkylamino ddNTPs using published methods (and as disclosed in U.S. Provisional Application No. 60/098,469 filed on August 31, 1998, the entire disclosure of which is hereby incorporated by reference herein). The labeled ddNTPs were purified by silica gel chromatography followed by ion exchange chromatography then reverse phase HPLC.

1.3 Experimental

All chemicals were purchased from Sigma, Aldrich, Fluka or Fisher Scientific unless stated otherwise. UV/visible spectra were recorded on a Perkin Elmer Lambda 20 UV/visible spectrophotometer in conjunction with Winlab™ software.

4-(propargylamido-4',5'-bissulfonatefluorescein)-N- α -*t*-butoxycarbonylphenylalanine (1)

4'-5'-bissulfono-5-carboxyfluorescein (100mg, 0.18mmol) was dissolved in DMF (4ml) then diisopropylethylamine (0.48ml, 15 eq.) and TSTU (65mg, 1.2eq.) added. The reaction mixture was stirred at room temperature for 1h. then 4-propargylamino-N- α -*t*-butoxycarbonylphenylalanine (69mg, 1.0eq) added. Stirring was continued for 3h. then the reaction mixture evaporated to dryness *in vacuo*. The product was isolated by reverse phase HPLC (C18, DeltaPak 15 μ , 100A, 50x300 μ m) eluting with 0-100% eluant B over 60 min (A = 0.1M TEAB, B = 50% MeCN/0.1MTEAB v/v, 100ml/min.). The product (retention time 37 min.) was evaporated to dryness *in vacuo* then coevaporated with MeOH (3x10ml) before

lyophilization (100mg, 65%). UV/vis (1M triethylammonium bicarbonate pH 8.8) 495nm (24670), 465nm (shoulder, 9634), 312nm (6708).

4-(propargylamido-4',5'-bissulfonatefluorescein)-phenylalanine- α -ammonium trifluoroacetate (2)

4-(propargylamido-4',5'-bissulfonatefluorescein)-N- α -*t*-butoxycarbonylphenylalanine (100mg, 0.12mmol) was treated with trifluoroacetic acid (10ml) for 15min. then evaporated to dryness *in vacuo*. The residue was coevaporated with toluene (3x10ml) then the product precipitated by the addition of Et₂O (50ml). The solid formed was collected by filtration, washed with cold Et₂O (3x50ml) then dried under high vacuum (100mg, 99%). R_f (tlc, iPrOH:NH₄OH:H₂O (6:3:1)=0.

General methodology for the attachment of rhodamine dyes to 2 (3)

4-(propargylamido-4',5'-bissulfonatefluorescein)-phenylalanine- α -ammonium trifluoroacetate 2 (0.1mmol) was dissolved in DMSO (1ml) then diisopropylethylamine (0.26ml, 15 eq.) and rhodamine-NHS active ester (1.5 eq.) added. The reaction mixture was stirred at room temperature for 16h, then evaporated to dryness *in vacuo*. The R110 analog was treated with triethylammonium bicarbonate solution (0.1M, 50ml) for 16h to remove the trifluoroacetimido protecting groups then the product purified by RP-HPLC using identical conditions to 1 unless stated. Retention times (BSFAM/R110 = 31min, BSFAM/R110 = 55min 0-100% B over 90 min, 100 ml/min., BSFAM/REG 54min 0-100%B over 90 min., 100ml/min, BSFAM/TAMRA = 52min 0-100% B over 90 min). All absorption spectra show the presence of both dyes.

General Methodology for Attachment of 3 to alkylamino-2',3'-dideoxynucleotide triphosphates (4).

The double dye cassette (1mmol) was dissolved in DMF (5ml) then disuccinimidyl carbonate (4eq.) and DMAP (4eq.) were added at -60°C. The reaction mixture was stirred at -30°C for 15 min. then a solution of aminoalkyl-ddNTP (0.67eq., Na₂CO₃/NaHCO₃ pH 8.5) added.

The reaction was stirred at room temperature for 1h. then applied directly to a SiO₂ gel column. The product was eluted with iPrOH:NH₄OH:H₂O (4:5:1 v:v:v) then evaporated to dryness *in vacuo* before subsequent purification by ion exchange chromatography then C18 reverse phase HPLC as for 1. Absorption spectra of each compound showed the presence of both dyes.

1.4 Comparative Electropherograms

One of the terminators (structure 4, Rhodamine =5-ROX and N = C) formed above was used in a sequencing reaction and run on a slab gel. The resulting electropherogram is shown in Figure 16 which provides an example of the increase in migration rate relative to sequence products of unincorporated bis-sulfonated fluorescein energy transfer terminators (and thermal breakdown products thereof) compared to the migration rate of the regular ET terminators.

2. An example of a negatively charged linker arm as applied to direct load

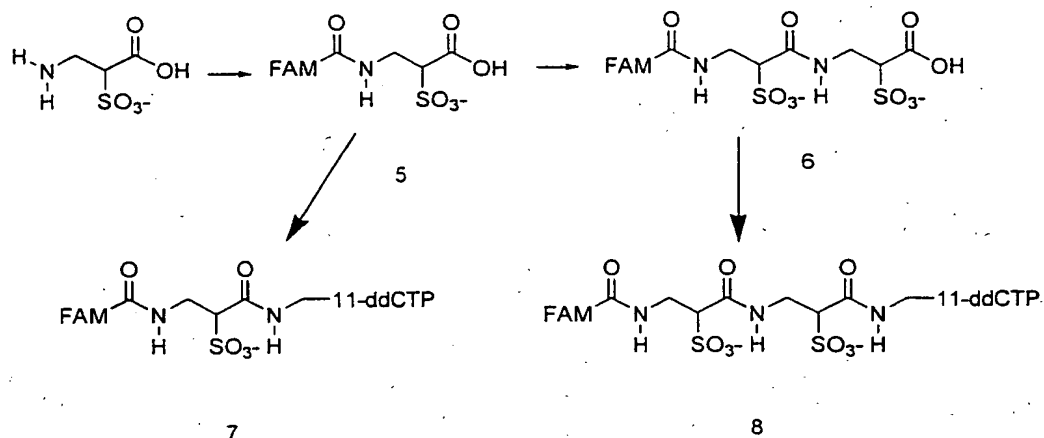
2.1 Background

By incorporation of a number of charged amino acids onto a fluorescent reporter, it is possible to synthesize a labeled ddNTP containing extra negative charge that alters the mobility of the degradative by-products observed in a sequencing reaction.

2.2 CHEMISTRY

In order to determine the amount of negative charge required to remove colored by-products from the sequence ladder, fluorescein was attached to α -sulfo- β -alanine to form 5.

Compound 5 was attached to a 11-ddCTP (11=number of atoms in linker arm) to form 7. A portion of 5 was attached to a second α -sulfo- β -alanine moiety to form 6 which was subsequently attached to 11-ddCTP to form 8. A control ddNTP containing regular FAM attached to 11-ddCTP was also synthesized. The structures were run in a single color sequencing reaction to determine the effect of the charge on mobility.



As fluorescein carries a net 1- charge, compound 7 is considered as overall 2- linker arm, compound 8 has an overall 3- linker arm charge.

2.3 Experimental

N-5-carboxamidofluorescein- α -sulfo- β -alanine (5)

α -sulfo- β -alanine (59mg, 0.35mmol) was dissolved in DMF (2ml) then diisopropylethylamine (0.9mol, 15eq) added followed by 5-FAM-NHS active ester (200mg, 1.2eq.). The reaction mixture was stirred at room temperature for 3h. then evaporated to dryness *in vacuo*. The residue was coevaporated with MeOH (10ml) then the product isolated by C18 RP HPLC (A=0.1MTEAB, B=0.1MTEAB, 50%MeCN v/v) eluting with 0-100%B over 90 min at 100ml/min. ^1H nmr (300MHz, CD_3OD); 1.27(t, 24H, $J=8.4\text{Hz}$, NCH_2CH_3), 3.05(q, 16H, $J=8.4\text{Hz}$, NCH_2CH_3), 3.95-4.05(m, 3H, $\text{CH}_2+\text{CHSO}_3$), 6.58(m, 3H, Ar-H), 6.85(d, 2H, $J=11.0\text{Hz}$, Ar-H), 7.30(d, 2H, $J=11.0\text{Hz}$, Ar-H), 8.02(d, 1H, $J=7.6\text{Hz}$, ArH), 8.45(s, 1H, Ar-H).

N-(N-5-carboxamidofluorescein- α -sulfo- β -alanine)amido- α -sulfo- β -alanine (6)

N-5-carboxamidofluorescein- α -sulfo- β -alanine (5, 50mg, 0.095mmol) was dissolved in DMF (3ml) then diisopropylethylamine (0.25ml, 15eq.) and TSTU (42mg, 1.5eq.) added. The reaction mixture was stirred at room temperature for 1h. then α -sulfo- β -alanine (24mg, 1.5eq.) added. Stirring was continued for 3h. then the reaction evaporated to dryness *in vacuo*. The product was isolated by ion exchange chromatography (mono-Q column, A=0.1M TEAB, 40%MeCN v/v, B=1.0M TEAB, 40%MeCN v/v, 0-50%B over 22min., 50-75%B from 22-50min. 75-100%B from 50-70 min., 4ml/min., retention time = 75-80min.) then C18 RP HPLC (A=0.1M TEAB B=0.1M TEAB/MeCN 50% v/v, 0-100%B over 90 min., 100ml/min, retention time = 33min.). R_f (PrOH6:ammonia3:water1 v/v/v) 0.34.

General Methodology for Attachment of modified dyes to alkylamino-2',3'-dideoxynucleotide triphosphates (7,8).

The modified dye (1mmol) was dissolved in DMF (5ml) then disuccinimidyl carbonate (4eq.) and DMAP (4eq.) were added at -60°C . The reaction mixture was stirred at -30°C for 15 min. then a solution of aminoalkyl-ddNTP (0.67eq., $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ pH 8.5) added. The reaction was stirred at room temperature for 1h. then applied directly to a SiO_2 gel column. The product was eluted with iPrOH: NH_4OH : H_2O (4:5:1 v:v:v) then evaporated to dryness *in vacuo* before subsequent purification by ion exchange chromatography then C18 reverse phase HPLC as for 1.

2.4 Results

Each labeled ddNTP was dissolved in sequencing buffer and subjected to several rounds of thermocycling. The products were separated on a sequencing gel and the electropherograms shown in Figure 17. Interpretation of the electropherogram provided the conclusion an overall 3- charge (i.e., structure 8) removed the colored by-products from the area of the electropherogram where true sequencing data would be obtained.

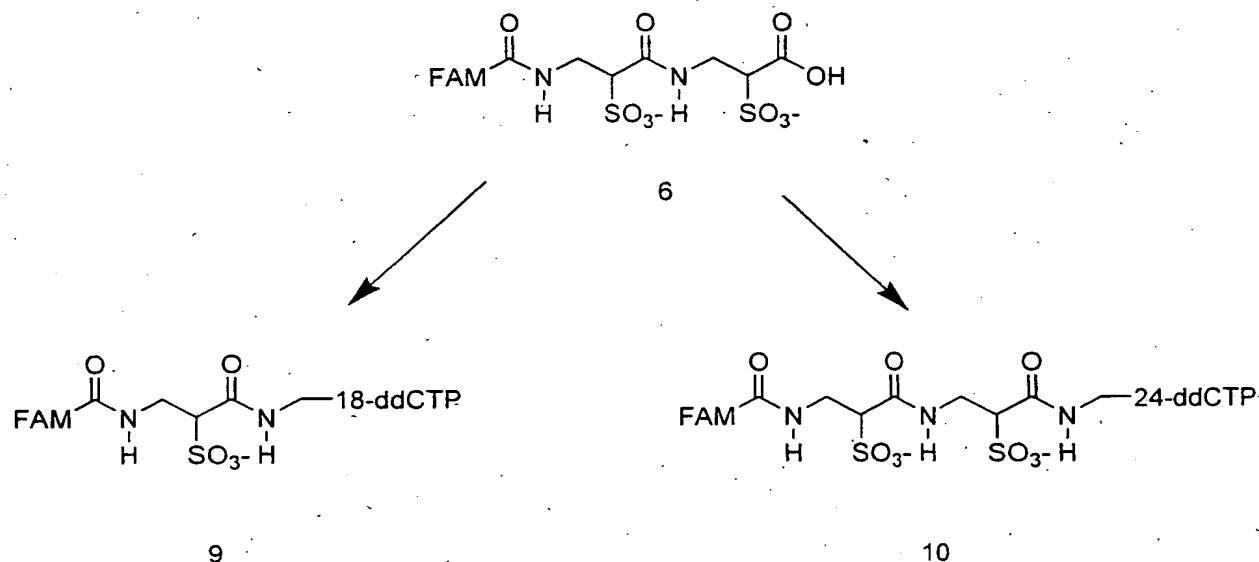
Figure 17 illustrates how the net negative charge of the dye labeled dideoxynucleotides affects their (and thermal breakdown products thereof) migration rate. As the net negative charge of the terminator increases, the migration rates of the various peaks seen (each of the peaks seen are either dye labeled dideoxynucleotides or thermal breakdown products thereof) increases (Figure 17). At an overall 3- charge (2- from linker, 1- from fluorescein) peaks are absent from the region of the electropherogram where true sequence data would normally be obtained.

3. Negatively charged extended linker arms as applied to direct load

3.1 Background

In order to improve the efficiency of incorporation of the modified terminator, a labeled terminator with a 3- charge on the linker arm was synthesized, this time containing an extended linker arm of 18 and 24 atoms.

3.2 Chemistry



3.3 Experimental

Compound 6, was attached to 18-ddCTP and 24-ddCTP using the standard protocol for attachment of labels to ddNTPs outlined in section 2.3. The method of purification was the same for 9 and 10.

Retention time of 9: Mono-Q™ ion exchange (47min)

Retention time of 10: Mono-Q™ ion exchange (42min)

C18 RP-HPLC (15min)

3.4 Sequencing Results

From the sequencing experiments it was clear that increasing the linker arm length improved incorporation of the terminator. This information, coupled to the presence of the 3- charge in the dye-linker structure led us to investigate rhodamine dyes with a 3- charged linker. This would permit four color sequencing.

As shown in Figure 18, it is possible to directly load a sequencing reaction with no clean-up procedure. Figure 18 shows no peaks resulting from unincorporated dye-labeled terminator in the sequence, thus demonstrating the utility of negatively charged terminators with respect to direct load sequencing.

3.5 Rhodamine Labeled Terminators Containing a 3- Linker Arm

The following chemistry was attempted to synthesize a set of four differently labeled terminators:

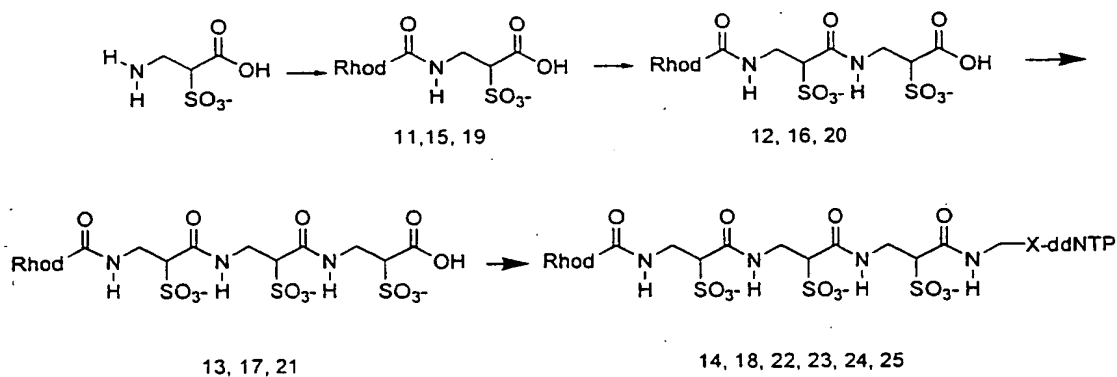


TABLE III

<u>Compound Nos.</u>	<u>Rhod</u>	<u>X</u>	<u>N</u>
11-14	REG	24	U
15-18	TAMRA	24	A
19-22	ROX	24	G
23	TAMRA	12	A
24	ROX	12	G
25	ROX	18	G

Rhod = rhodamine label, X = length of linker arm, N=base

3.6 Experimental

Compounds 11, 15, 19 were synthesized according to the method outlined for 5.

Compounds 12, 13, 16, 17, 17, 21 according to the method outlined for 6.

Compounds 14, 18, 22-25 according to the general methodology for attachment of modified dyes to alkylamino-2',3'-dideoxynucleotide triphosphates (7,8).

3.7 Results and Discussion

The labeled triphosphates 14, 18, 22 were used in a direct load sequencing experiment.

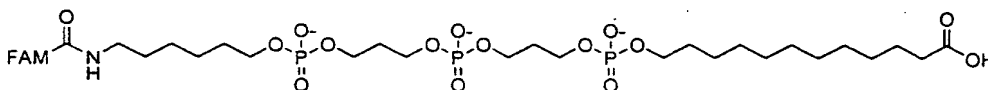
Compound 14 in a direct load experiment showed no breakdown products and with TSII and

TaqERDAFY. Compounds 18 and 22 gave very dark sequencing bands and were observed to be forming an unexpected aggregate (as observed in the emission spectrum). The compounds also produced large colored blobs on a sequencing gel which interfered with interpretation of the sequence.

In order to overcome the aggregation effect, structures 23-25 were synthesized to investigate the effect of a shorter linker arm. Compound 23 has been shown to yield a clean sequence, 24 and 25 are awaiting testing. Structures 23-25 all have the expected rhodamine emission spectrum hence it appears that the aggregation problem may have been overcome.

4 Other examples of negatively charged linker arms

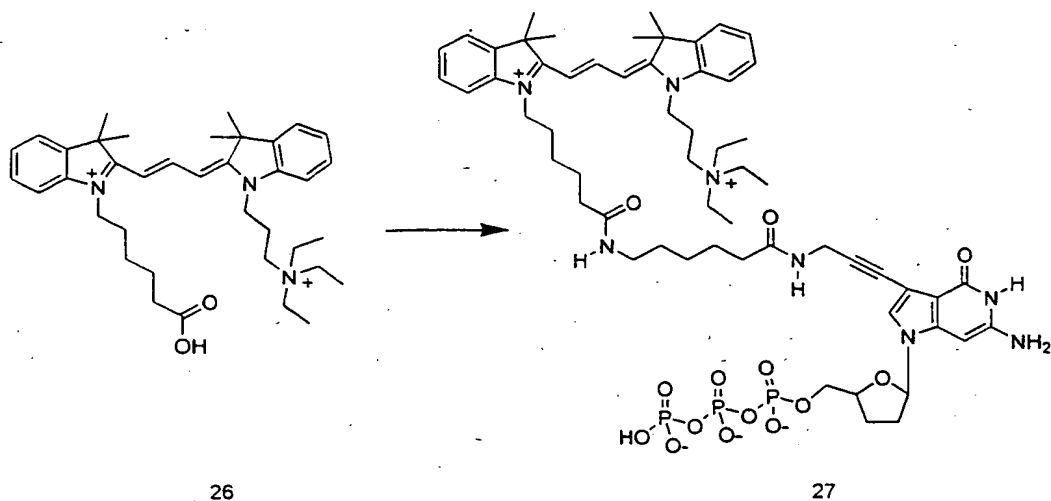
Other negatively charged linker arms have been synthesized and studied for example the phosphodiester structure shown below. The product was synthesized using phosphoramidite chemistry however it could also be synthesized via H-phosphonates, phosphoroimidazolides, or phosphotriester chemistry.



5. Examples of Terminators with Positively Charged Reporters

5.1 Background

In order to study positively charged structures, the following labeled terminator was synthesized.



5.2 Experimental

Compound 26 (10mg, 0.0134mmol) was dissolved in DMF (1ml) then diisopropylethylamine (23 μ l, 10eq.) added followed by PyBOP (14mg, 2.0eq.). The reaction mixture was stirred at room temperature for 15min. then a solution of 11-ddGTP (0.0083mmol, Na₂CO₃-NaHCO₃ pH 8.5) added in one portion. The reaction mixture was stirred at room temperature for 3h. then applied directly to a silica gel column. The product was eluted with iPrOH:NH₄OH:H₂O (6:3:1 v/v/v) then purified by ion exchange chromatography (as for 6) followed by C18 RP-HPLC (1.75 μ mol yield, 21%).

5.3 Sequencing Results

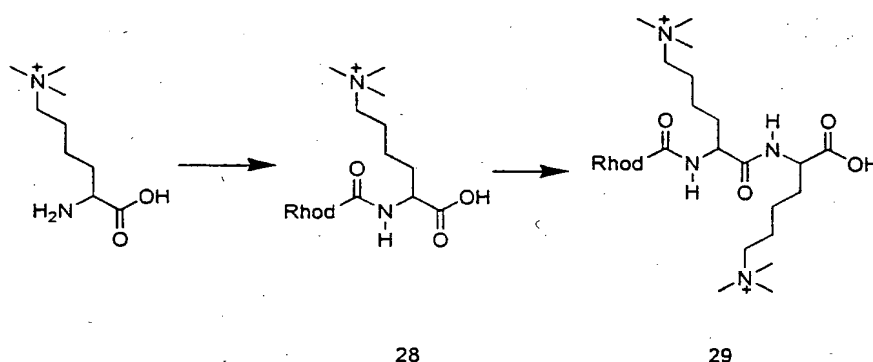
The electropherogram shown in Figure 19 was obtained when 27 was used in a sequencing reaction. The +2 charged terminator was used in a sequencing reaction and loaded directly on to a slab gel. The same experiment was repeated, however the reaction mixture was treated with phosphatase prior to loading on a gel to remove phosphates from the unincorporated dye-labeled dideoxynucleotides remaining in the reaction mixture. This leaves all terminator derived products with an overall positive charge causing them to migrate in the opposite direction as the sequence products during electrophoresis. It is clear from the

electropherogram that the colored by-products are absent from the sequence when phosphatase is used to break down the terminator products.

6. Positively charged extended linker arms as applied to direct load

6.1 Chemistry

Another example of dyes attached to a positively charged linker arm is shown below;



In this example, the rhodamine dye R6G is attached to ϵ -N,N,N-trimethyllysine which contains a formalized positive charge from the ϵ quaternary amine. The product (28) can be further modified to yield a +2 linker arm (29) by reaction with a further molecule of the charged amino acid. Further reaction(s) would generate the desired charged structure.

6.2 Experimental

α -N-(5-carboxamidorhodamine6G)- ϵ -N,N,N-trimethyllysine (28)

ϵ -N,N,N-trimethyllysine (68mg, 30.0mmol) was dissolved in DMF (6ml) then diisopropylethylamine (0.5ml, 10eq.) added followed by R6G-NHS active ester (200mg, 1.2eq.). The reaction mixture was stirred at room temperature for 16h. then evaporated to dryness *in vacuo*. The product was isolated by C18 RP HPLC (A=0.1M TEAB, B=0.1MTEAB/50%MeCN, 0-100%B over 50 min., 100ml/min). Retention time = 44min.

α -(α' -N-(5-carboxamidorhodamine6G)- ϵ' -N,N,N-trimethyllysine)- ϵ -N,N,N-trimethyllysine (29)

α -N-(5-carboxamidorhodamine6G)- ϵ -N,N,N-trimethyllysine 28 (100mg, 0.15mmol) was dissolved in DMF (5ml) then diisopropylethylamine (0.3ml, 1.5eq.) and TSTU (67mg, 1.5eq.) added. The reaction mixture was stirred at room temperature for 1h. then ϵ -N,N,N-trimethyllysine (50mg, 1.5eq.) added. The solution was stirred for a further 3h. then the reaction mixture was evaporated to dryness *in vacuo*. The product was isolated by C18 RP HPLC (A=0.1M TEAB, B=0.1MTEAB/50%MeCN, 0-100%B over 90 min., 100ml/min). Retention time = 60min.

TABLE IV

Abbreviations

<u>Abbreviation</u>	<u>Definition</u>
ddNTP	2'-3'-dideoxynucleoside triphosphate
ET	Energy Transfer
TSTU	2-Succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate
PyBOP	Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
DMF	N,N-dimethylformamide
RP HPLC	Reverse Phase High Performance Liquid Chromatography
Et ₂ O	Diethyl ether
DMSO	Dimethyl sulfoxide
TEAB	Triethylammonium bicarbonate
MeCN	Acetonitrile
iPrOH	Isopropanol
NH ₄ OH	Ammonium Hydroxide
BSFAM	4',5' Bis-sulfono-5-carboxyfluorescein
R110	Rhodamine 110
REG or R6G	Carboxyrhodamine6G
TAMRA	Tertamethylrhodamine
ROX	Carboxy-X-rhodamine
DMAP	4-dimethylaminopyridine
11-ddGTP	2',2'-dideoxyguanosine triphosphate with an 11 atom linker arm
NHS	N-hydroxysuccinimide

Although various embodiments of the instant invention are described in detail above, the instant invention is not limited to such specific examples. Various modifications will be readily apparent to one of ordinary skill in the art and fall within the spirit and scope of the following appended claims.

CLAIMS

What is claimed is:

1. A purified recombinant thermostable DNA polymerase comprising the amino acid sequence set forth in Figure 2.
2. A purified recombinant thermostable DNA polymerase comprising an amino acid substitution corresponding to the substitution E681R in the amino acid sequence listing of Figure 2.
3. An isolated nucleic acid that encodes a thermostable DNA polymerase, wherein said nucleic acid consists of the nucleotide sequence corresponding to the amino acid sequence set forth in Figure 2.
4. A recombinant DNA vector that comprises the nucleic acid of Claim 3.
5. A recombinant host cell transformed with the vector of Claim 4.
6. The recombinant host cell of Claim 5 that is *E. coli*.
7. A method of sequencing DNA comprising the step of generating chain terminated fragments from the DNA template to be sequenced with the DNA polymerase of Claim 1 in the presence of at least one chain terminating agent and one or more nucleotide triphosphates, and determining the sequence of said DNA from the sizes of said fragments.
8. A method according to Claim 7, wherein the chain terminating agent comprises a labeled nucleic acid terminator having a net positive or a net negative charge.
9. A method for synthesizing a fluorescently labeled polynucleotide, said method comprising the step of mixing a DNA polymerase according to Claim 1 with a primed template.
10. A method according to Claim 9, wherein the primed template is a primed template in a chain termination sequencing reaction.

11. A method according to Claim 9, wherein the primed template is a primed template in a polymerase chain reaction.
12. A kit for fluorescently labeling a polynucleotide, the kit comprising a DNA polymerase according to Claim 1 and a fluorescently labeled nucleotide.
13. A kit according to Claim 12, wherein the fluorescently labeled nucleotide comprises a nucleic acid terminator having a net negative or a net positive charge.
14. A kit for sequencing DNA comprising the DNA polymerase of Claim 1.
15. A purified recombinant thermostable DNA polymerase comprising the amino acid sequence set forth in Figure 3.
16. A DNA polymerase comprising an amino acid substitution corresponding to the substitution E681R in the amino acid sequence listing of Figure 3.
17. An isolated nucleic acid that encodes a thermostable DNA polymerase, wherein said nucleic acid consists of the nucleotide sequence corresponding to the amino acid sequence set forth in Figure 3.
18. A recombinant DNA vector that comprises the nucleic acid of Claim 17.
19. A recombinant host cell transformed with the vector of Claim 18.
20. The recombinant host cell of Claim 18 that is *E. coli*.
21. A method of sequencing DNA comprising the step of generating chain terminated fragments from the DNA template to be sequenced with the DNA polymerase of Claim 16 in the presence of at least one chain terminating agent and one or more nucleotide triphosphates, and determining the sequence of said DNA from the sizes of said fragments.
22. A method according to Claim 21, wherein the chain terminating agent comprises a labeled nucleic acid terminator having a net positive or a net negative charge.

23. A method for synthesizing a fluorescently labeled polynucleotide, said method comprising the step of mixing a DNA polymerase according to Claim 16 with a primed template.
24. A method according to Claim 23, wherein the primed template is a primed template in a chain termination sequencing reaction.
25. A method according to claim 23, wherein the primed template is a primed template in a polymerase chain reaction.
26. A kit for fluorescently labeling a polynucleotide, the kit comprising a DNA polymerase according to Claim 16 and a fluorescently labeled nucleotide.
27. A kit according to Claim 26, wherein the fluorescently labeled nucleotide comprises a nucleic acid terminator having a net negative or a net positive charge.
28. A kit for sequencing DNA comprising the DNA polymerase of Claim 16.

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Taq DNA polymerase:

5 MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGF
AKSLLKALKEDGDAVIVVFDKAPSFRHEAYGGYKAGRAPTPEDFPRQ
LALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADK
DLYQLLSDRIHVLHPEGYLITPAWLWEKYGLRDPDQWADYRALTGDES
DNLPGVKGIGECTARKLLEEWGSLEALLKNLDRKPAIREKILAHMDD
10 LKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGL
LESPKALEEAPWPPPEGAFVGVLSRKEPMWADLLALAAARGGRVHR
APEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDP
SNTTPEGVARRYGGEWTEEAGERAAALSERLFANLWGRLEGEERLLWL
YREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRL
15 AGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAH
PIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDE
NLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMS
AHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLF
20 GRRRYVPDLEARVYKSVREAAERMAFNMPVQGTAAADLMKLA MVKLP
RLEEMGARMLLQVHDELVLEAPKERA EAVARLAKEVM EGVYPLAVPL
EVEVGIGEDWLSAKE

Fig. 1

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Taq Δ 271/F272M/F667Y/E681R DNA polymerase:

5 MLERLEFGSLLHEFGLLESPKALEEAPWPPPEGAFVGVLSRKEPMWA
DLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLG
LPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAAALSERLFA
NLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSL
EVAEEIARLEAEVFRLAGHPFNLSRDQLERVLFDDELGLPAIGKTEKTG
10 KRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRL
HTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVAL
DYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPL
MRRAAKTINYGVLYGMSAHRLSQRLAIPYEEAQAFIERYFQSFPAKVR
WIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFNMP
15 VQGTAAADLMKLAMVKLFPRLEEMGARMLLQVHDELVLEAPKERA
VARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKE

Fig. 2



3/19Taq D18A/E681R/F667Y DNA polymerase:

5 MRGMLPLFEPKGRVLLVAGHHLAYRTFHALKGLTTSRGEPVQAVYGF
AKSLLKALKEDGDAVIVVFDKAPSRHEAYGGYKAGRPTPEDFPRQ
LALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADK
DLYQLLSDRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDES
DNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLKPAIREKILAHMDD
10 LKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGL
LESPKALEEAPWPPPEGAFVGVLSRKEPMWADLLALAAARGGRVHR
APEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDP
SNTTPEGVARRYGGEWTEEAGERAAALSERLFANLWGRLEGEERLLWL
YREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRL
15 AGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAH
PIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDE
NLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINYGVLYGMS
AHRLSQRLAIPYEEAQAFIERYFQSFQKVRAWIEKTLEEGRRRGYVETL
20 FGRRRYVPDLEARVKS VREAAERMAFNMPVQGTAAADLMKLAMVKLF
PRLEEMGARMLLQVHDELVLEAPKERAEEAVARLAKEVMEGVYPLAVP
LEVEVGIGEDWLSAKE

Fig. 3

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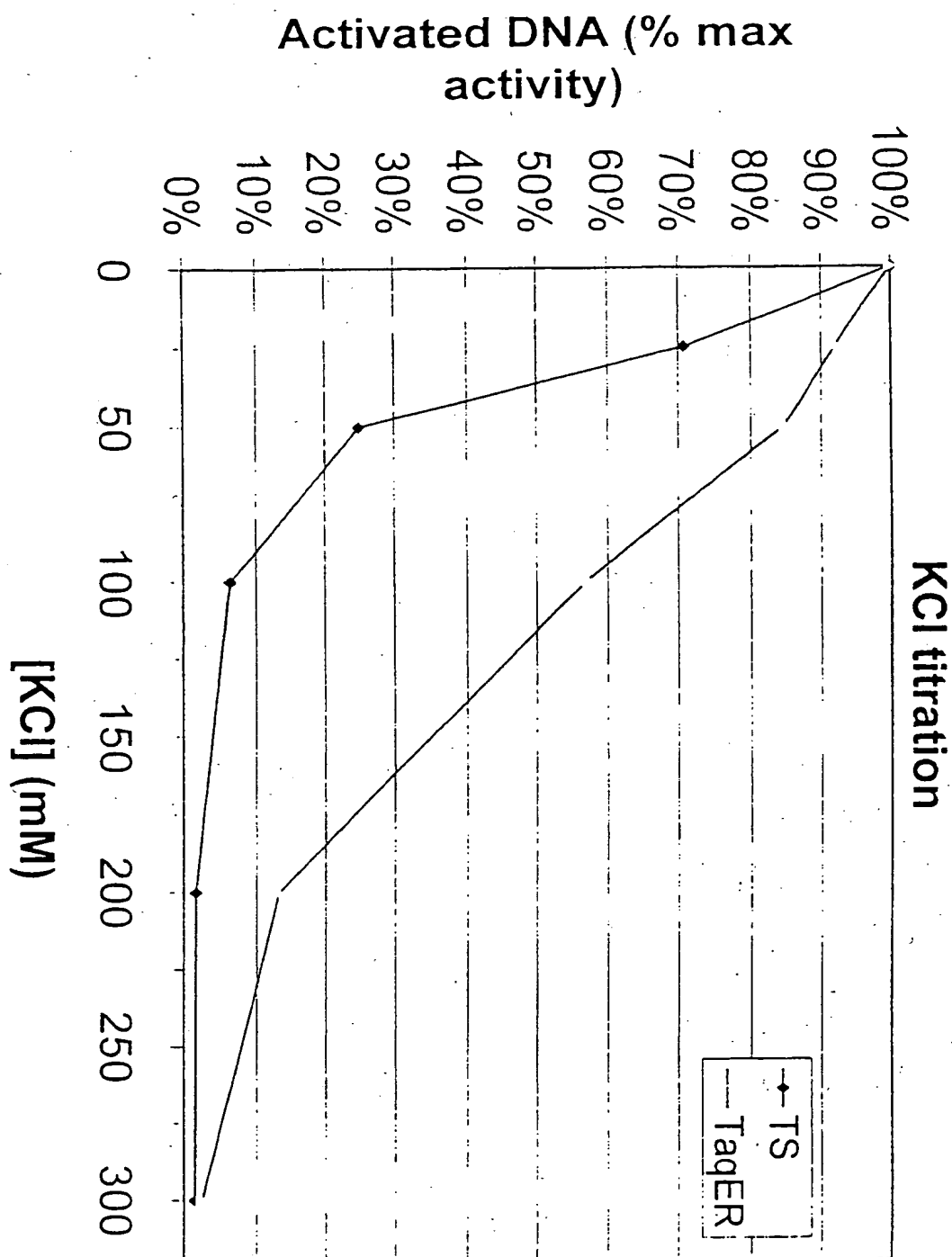
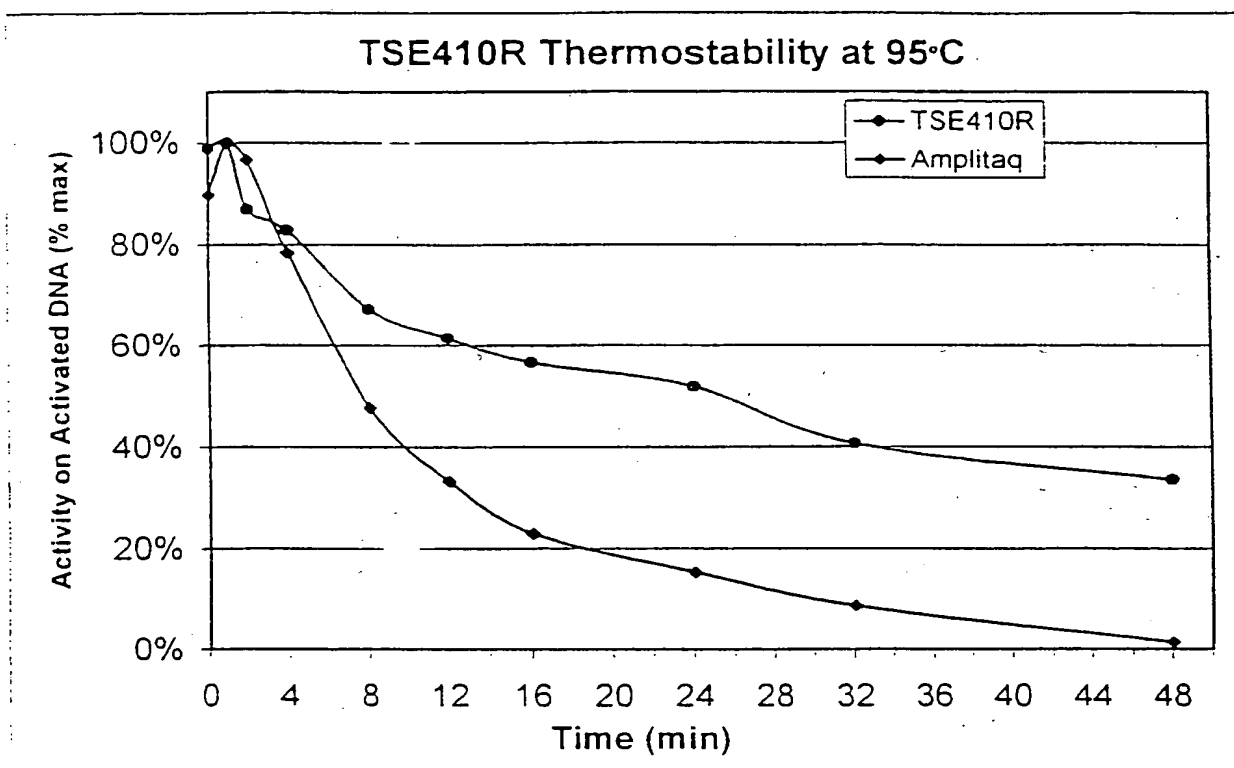


Fig. 4

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**Fig. 5**

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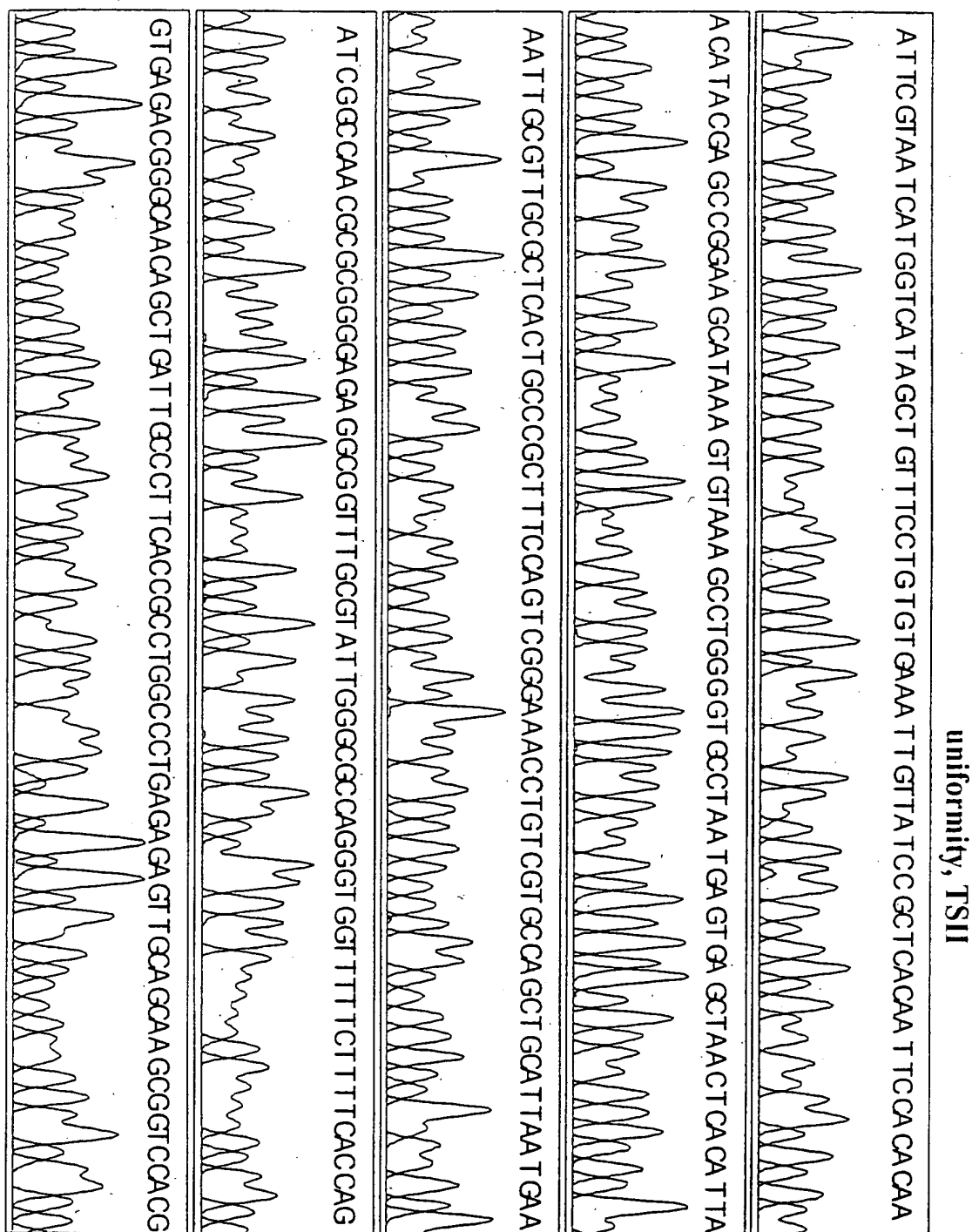


Fig. 6

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uniformity, TSII ER

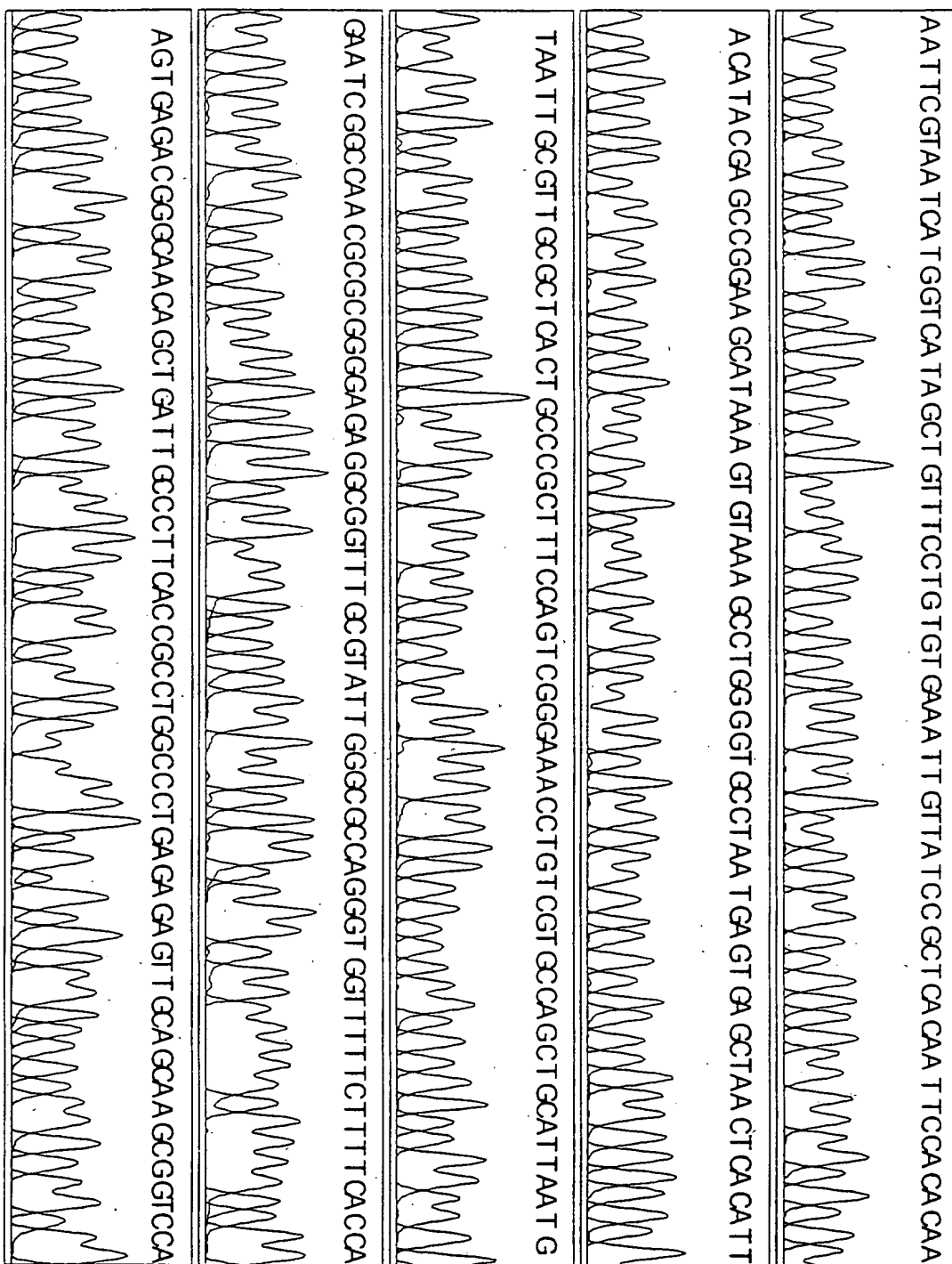


Fig. 7

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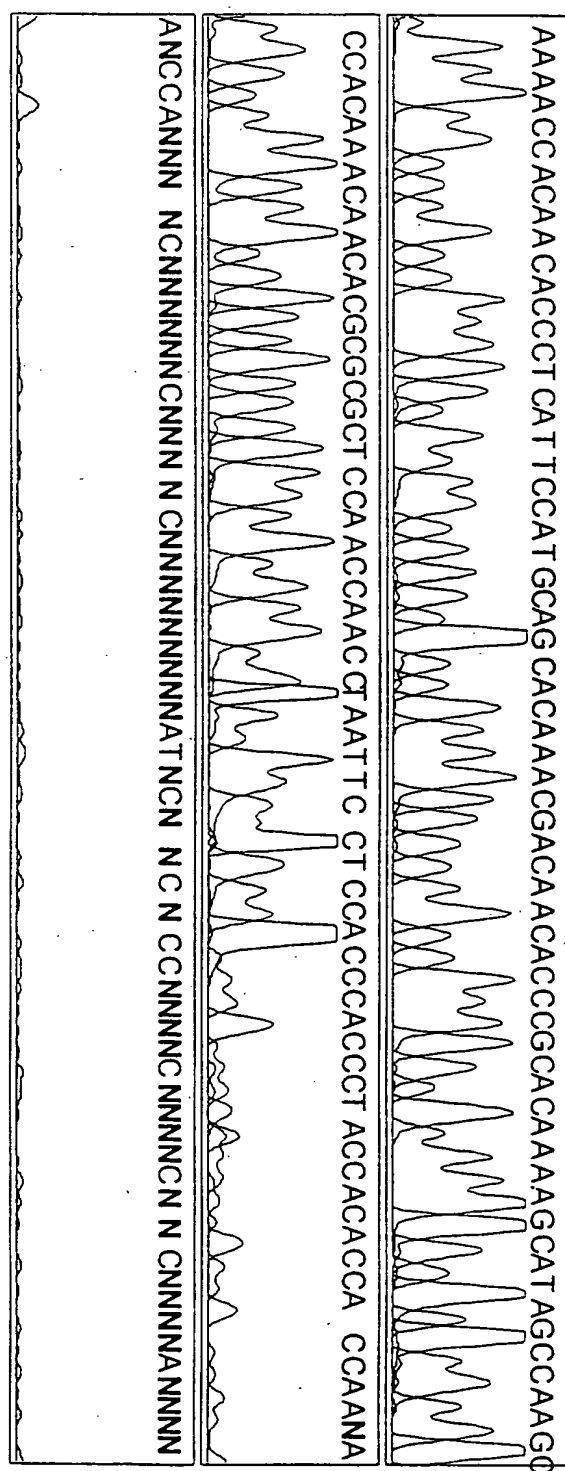


Fig. 8

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Difficult template, TSII ER

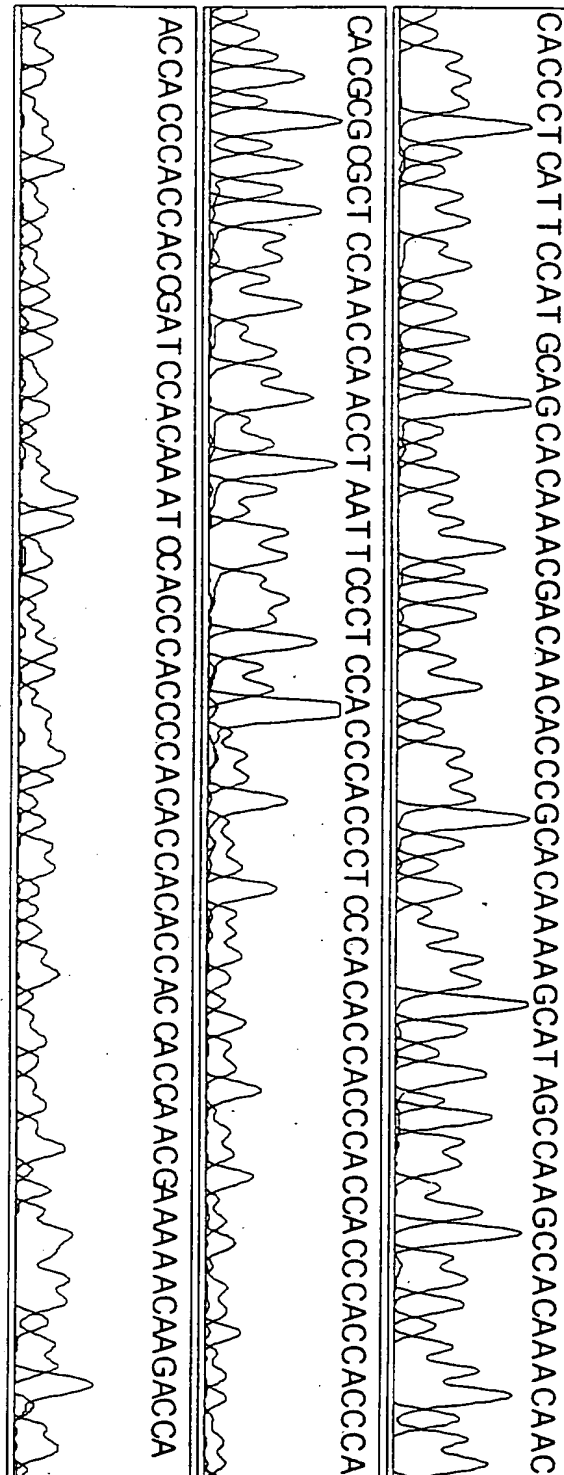


Fig. 9

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Fig. 10a

DNA sequence using TSII.
Figure shows example of
a strong \ relative to following
A's.

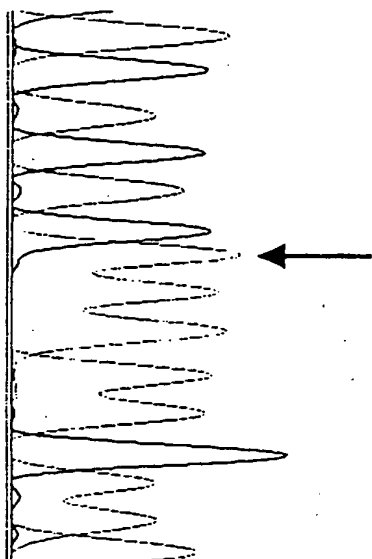
T G T G T G A H A T T G T T A
100



T G T G T G A H A T T G T T A
100

Fig. 10b

Same sequence using an E to R
Polymerase. The strong \ relative
to following \ 's is eliminated.



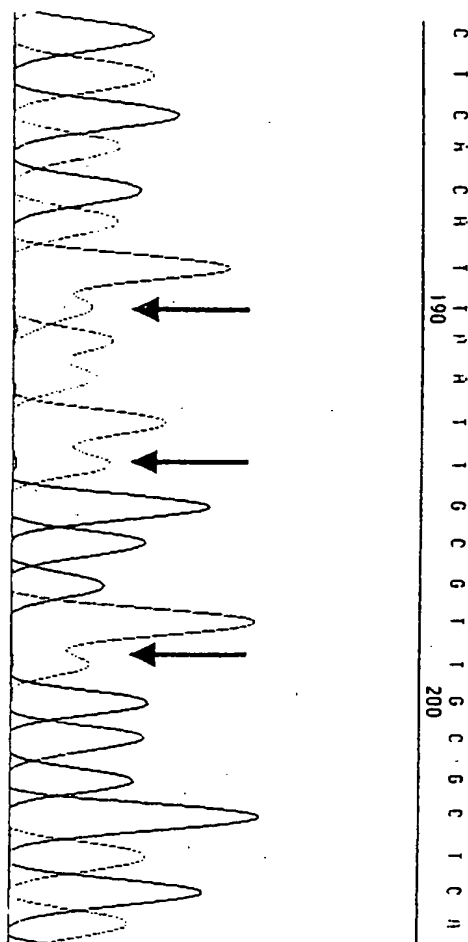
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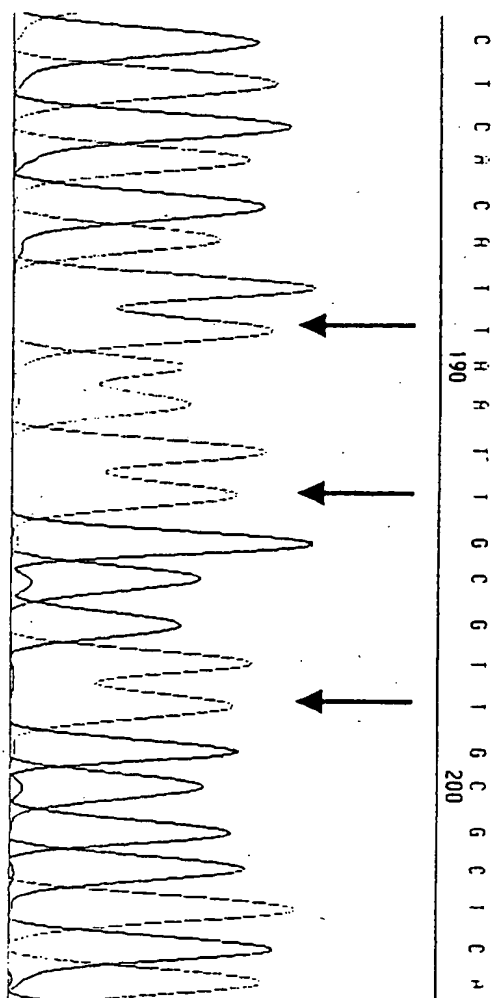
11/19

Fig. 11a

DNA sequence using TSL.
Figure shows multiple
examples of weak 'I's after
preceding 'I's.

**Fig. 11b**

Same sequence using an E to R
Polymerase. The weak 'I' after a
preceding 'I' is eliminated.



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Fig. 12a

DNA sequence using TSL.
Figure shows example of
a weak G after preceding \.

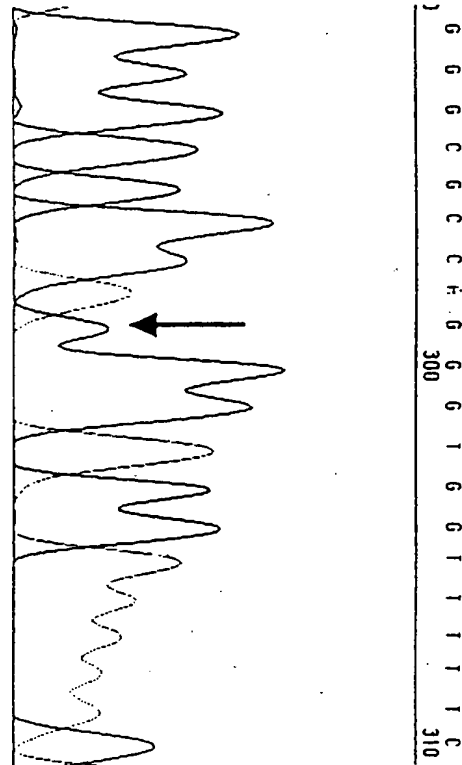
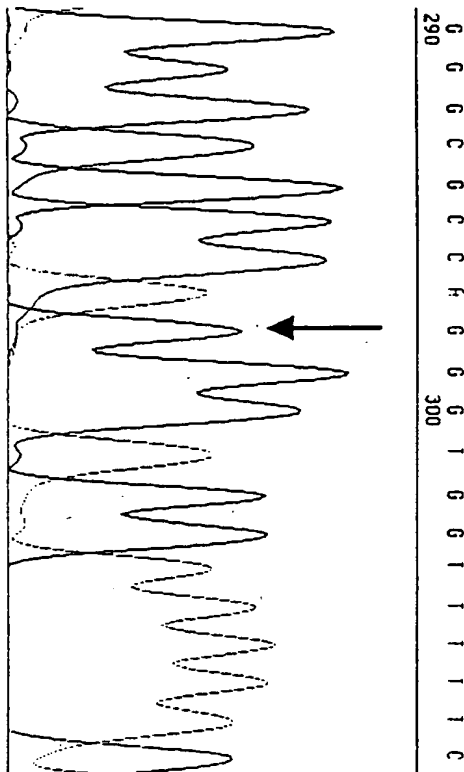


Fig. 12b

Same sequence using an E to R
Polymerase. The weak G after
preceding \ is eliminated.





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681

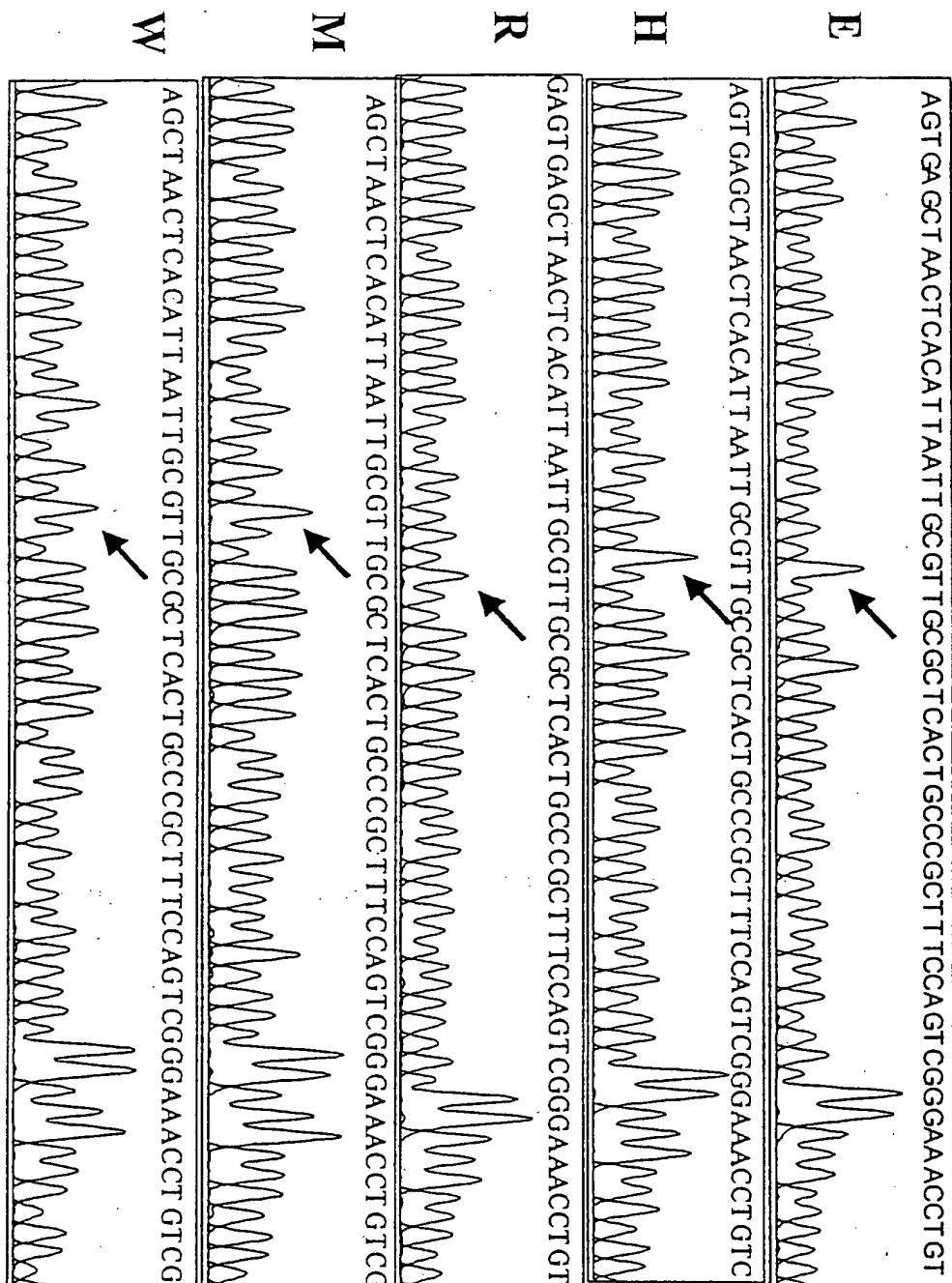


Fig. 13

14/19

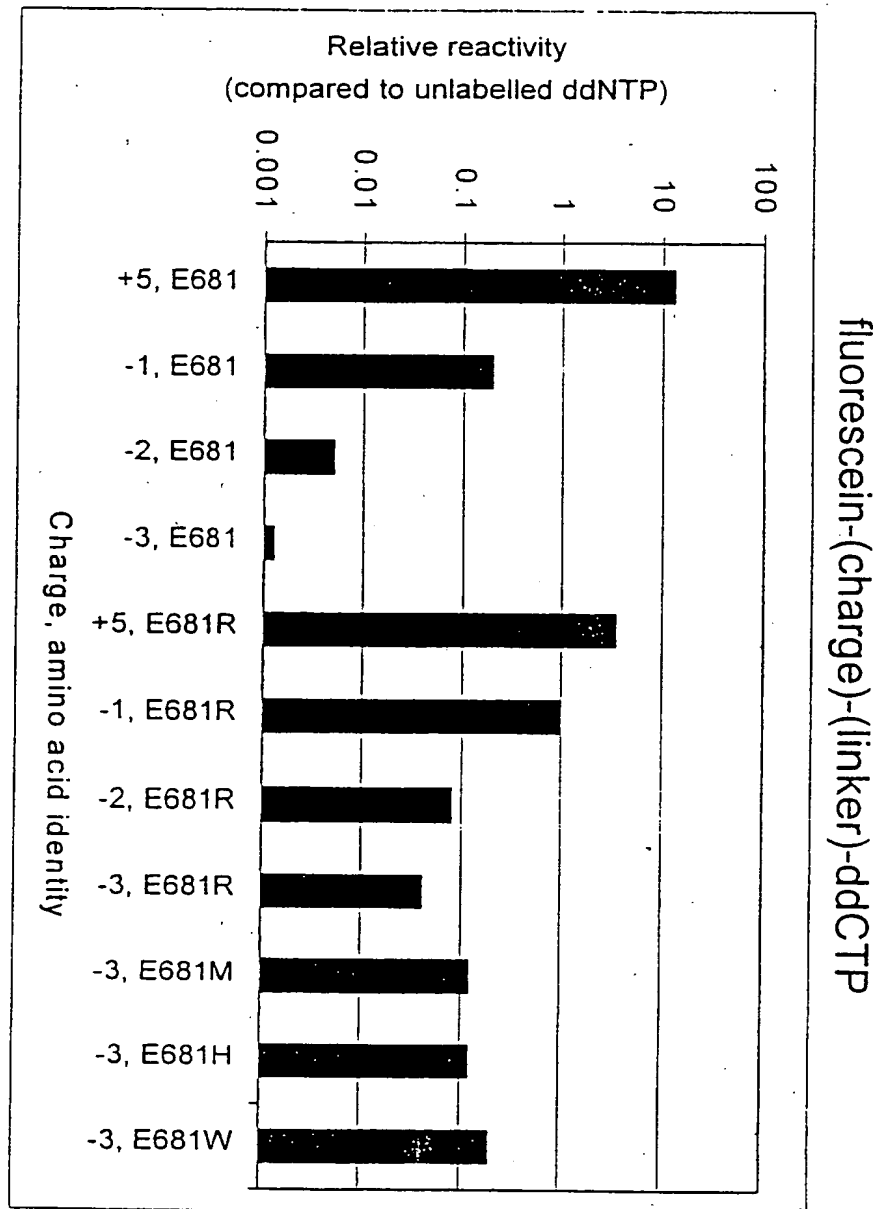


Fig. 14

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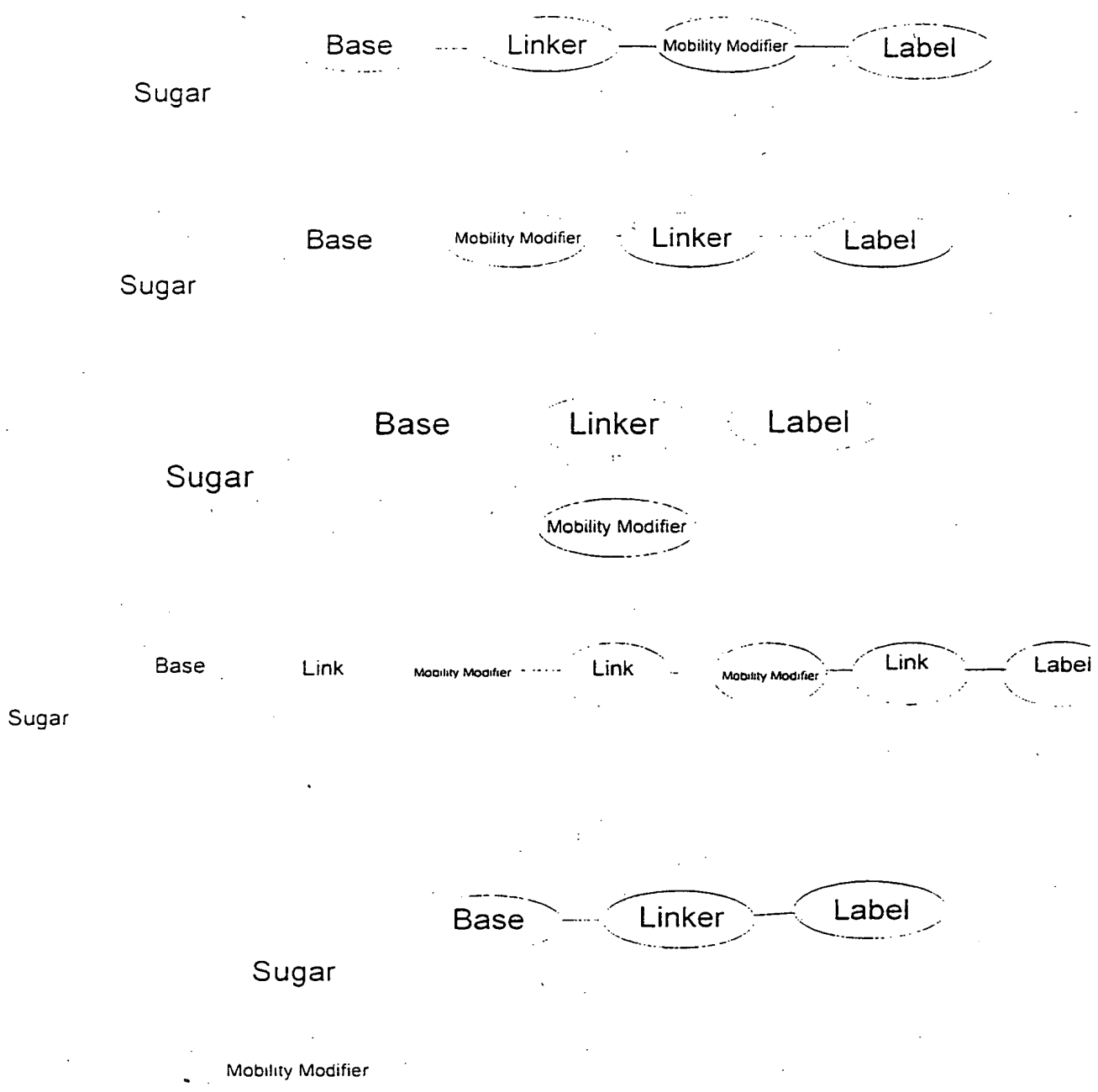


Fig. 15

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Fig. 16a

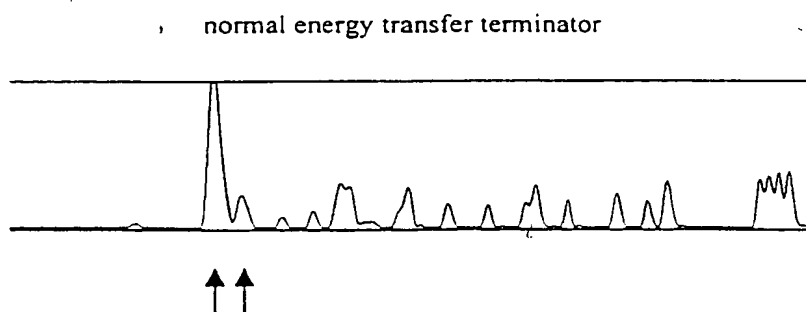
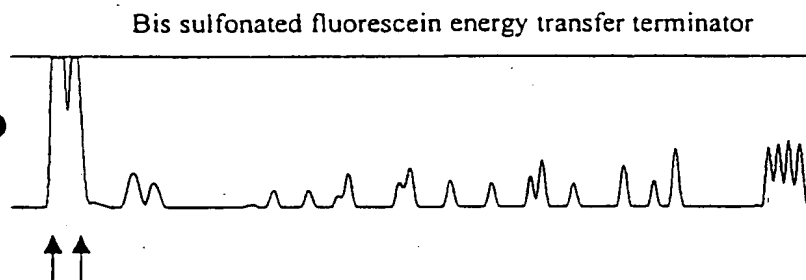


Fig. 16b



Faster

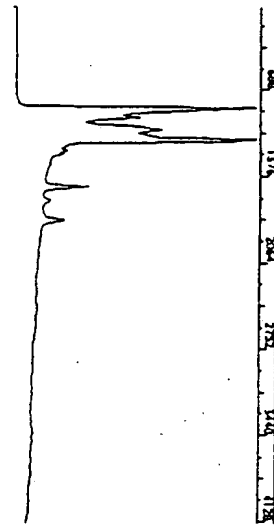
Slower

Comparison of Regular v. Bis-sulfonated Fluorescein ET Terminators



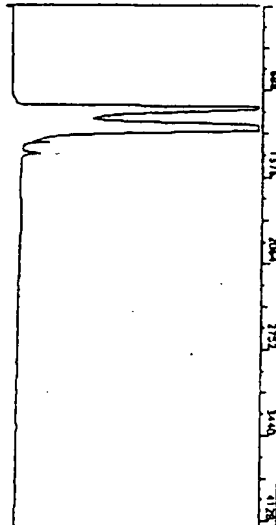
17/19

Fig. 17a



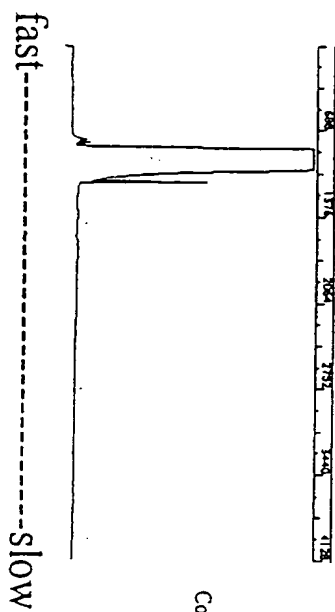
FAM-11-ddCTP

Fig. 17b



Compound 7

Fig. 17c

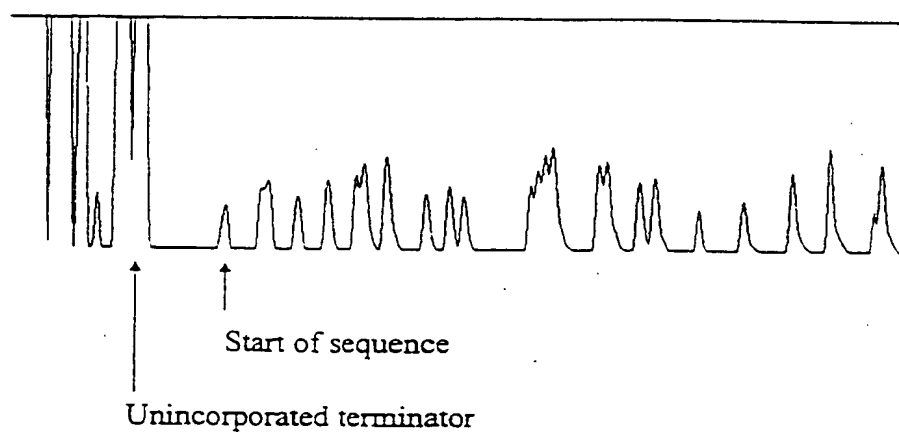


Compound 8

fast-----slow

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Net -3 charge terminator (10) reaction, directly loaded

**Fig. 18**

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Fig. 19a

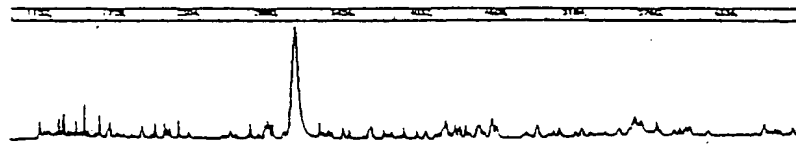
net +2 charge terminator,
no phosphatase treatment

Fig. 19b

net +2 charge terminator,
after phosphatase treatment